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Fitness consequences of the combined effects of veterinary and agricultural pesticides on a non-target insect

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HM, RK, SL, and WUB conceived the experiments. HM, RK, JR, and NG carried out the experiments. RK and HM did the statistical analyses and wrote the paper with input from all co-authors.

Abstract

Pesticides and veterinary products that are globally used in farming against pests and parasites are known to impact non-target beneficial organisms. While most studies have tested the lethal and sub-lethal effects of single chemicals, species are exposed to multiple contaminants that might interact and exacerbate the toxic responses of life-history fitness components. Here we applied experimentally a widespread ecotoxicological scenario in nature where non-target dung communities are exposed to both cattle parasiticides during the larval stage and agricultural insecticides during their adult life. We assessed the independent and combined consumptive effects of ivermectin (control, 12, and 24 μg kg^{-1} wet dung) and spinosad (control and sprayed 0.02 % ml kg^{-1}) on juvenile life-history and adult reproductive traits of the widespread yellow dung fly (*Scathophaga stercoraria*; Diptera: Scathophagidae). Larval exposure to ivermectin prolonged development time and reduced egg-to-adult survival, body size, and the magnitude of the male-biased sexual size dimorphism. The consumption by the predatory adult flies of spinosad-contaminated prey showed an additional, independent (from ivermectin) negative effect on female clutch size, subsequent egg hatching success, but not on the body size and sexual size dimorphism of their surviving offspring. However, there were interactive synergistic effects of both contaminants on offspring emergence and body size. Our results document negative effects of the combination of different chemicals on fitness components of a dung insect, highlighting transgenerational effects of adult exposure to contaminants for their offspring. These findings suggest that ecotoxicological tests should consider the combination of different contaminants for more accurate eco-assessments.

Keywords: Body size; Contamination; Ivermectin; Pollution; Reproductive success ; Spinosad.

Introduction

The sources of contamination can come from both the biotope and its biota (e.g. food and prey). If contaminants in polluted habitats persist for a long time (Lumaret et al., 2012), they can accumulate across trophic levels through the food chain (Cabana and Rasmussen, 1994; Jamieson et al., 2017), referred to as bioaccumulation: the higher the trophic level, the higher the concentration of contaminants. Such toxic compounds may have drastic consequences on individual fitness with further potential repercussions on human health (Margni et al., 2002; Blair et al., 2015).

Farmers have globally used pesticides and veterinary products to protect their crops and livestock against diseases, pests, and parasites (Boxall et al., 2004; Guedes et al., 2016), thereby causing local pollution of the environment with large-scale impacts. These products, which can spread and remain as residues in the environment, are usually not specifically targeted to any undesirable organisms and thus also affect non-target beneficial communities that can play a crucial role in the environment (Desneux et al., 2007). As a consequence, many ecosystem functions and services, such as pollination and biodegradation, may be disrupted (Pascoal et al., 2003; Medina et al., 2007), impacting the environment and the economy (Potts et al., 2010).

Among the beneficial organisms, the diverse community of insects and other invertebrates that decompose and recycle the nutrients of dung is particularly threatened by chemical applications of pesticides and other pharmaceutical products (Lumaret et al., 2012; Floate et al., 2016; Alvarado et al., 2018). After spending the larval stage in dung, the adult insects often occupy agricultural landscapes and are further affected by pesticides applied to crops to kill insects or herbs. The predators of this community (e.g. certain flies and beetles) and other organisms (e.g. wasps, lizards or birds) will prey on contaminated prey and thus accumulate toxins from various

77 sources (Hallmann et al., 2014). Although this scenario is widespread in nature (Edwards, 2013;
78 Gilburn et al., 2015), we still have limited knowledge of the fitness consequences of different
79 sources of contaminations for the biota. While two pesticides could affect individuals additively
80 (total effect = A+B), they could also interact and show synergistic effects (total effect > A+B) or
81 antagonistic effects (total effect < A+B). However, the numerous potential combinations of
82 multiple chemical substances in the wild complicates the assessment of such combined risks.
83 Nonetheless, using some commonly applied substances provides insights into their potentially
84 widespread additive and interactive effects on biota.

85 Ivermectin is an antiparasitic drug that is widely applied to cattle against nematodes and ticks
86 (Alegría-López et al., 2015). This medication is regularly excreted with the dung of the treated
87 animal, and can last for months in the habitat (Errouissi et al., 2001), affecting non-target
88 communities of arthropods, especially those living in the soil and animal feces (Römbke et al.,
89 2010; Lumaret et al., 2012). The effect of ivermectin residues in the dung, and the high
90 sensitivity of the dung community to it, are well documented (Madsen et al., 1990; Strong and
91 James, 1993; Römbke et al., 2009; Römbke et al., 2010; Blanckenhorn et al., 2013; Verdú et al.,
92 2015; Conforti et al., 2018). The half-life degradation of ivermectin has been reported between
93 93-240 days during winter and 7-14 days during summer (Halley et al., 1989). Besides
94 augmenting mortality, ivermectin has additional non-lethal impacts on life-history traits, such as
95 delaying development and reducing body size, in sepsid dung flies (Blanckenhorn et al., 2013)
96 and several dung beetles (Errouissi et al., 2001; González-Tokman et al., 2017). This in turn
97 impedes their mating behavior as adults, reduces reproductive success even at low ivermectin
98 concentrations (Conforti et al., 2018), slows down the locomotion of dung beetles (Verdú et al.,

2015), ultimately disturbing the natural process of dung degradation (Madsen et al., 1990; Römbke et al., 2010; Lumaret et al., 2012; Floate et al., 2016).

Spinosad is a natural insecticide extracted from soil bacteria (Lumaret et al., 2012). This insecticide has neurotoxic properties acting as a contact and digestive poison. It is widely used against crop pests, flies and mosquitoes. Although spinosad has been shown to be effective against insect pests like caterpillars (Sparks et al., 1998), beetles (McLeod et al., 2002), and the spotted wing fruit fly *Drosophila suzukii* (Van Timmeren and Isaacs, 2013), it also affects other non-target insects through direct contact or food such as nectar and prey (Desneux et al., 2005; Badji et al., 2007; Guedes et al., 2016). Many studies have demonstrated spinosad impacts on insect communities, but only few studies have tested its interaction with other dominant contaminants such as pyriproxyfen (in the mosquito *Aedes aegypti*; Darriet and Corbel, 2006), showing strong synergistic effects.

The yellow dung fly is a long-established model organism in evolutionary biology (Blanckenhorn, 1997; Ward, 2000) and ecotoxicology (Römbke et al., 2009). It is a convenient organism to test ecotoxicological questions because it is widespread and easy to rear in the laboratory, has a short life cycle (3 to 4 weeks of larval development: Blanckenhorn, 1998; Blanckenhorn and Henseler, 2005), and is sensitive to pharmaceuticals used for livestock treatment (Strong and James, 1992, 1993; Römbke et al., 2009). As a consequence, *S. stercoraria* has been approved as a test species for the evaluation of the toxicity of drug residues in dung by international regulating authorities (OECD 2008). Several studies have assessed the role of dung contamination by ivermectin on the fitness and life-history traits of this species (Strong and James, 1993; Römbke et al., 2009; West and Tracy, 2009). For instance, ivermectin decreased the survival rate of larvae by 50% within 48h at a concentration of 0.036 ppm (wt/wet

weight), delayed larval development and reduced body size and reproductive success (Römbke et al., 2010), affected wing morphology (Strong and James, 1993) and the fly's immune system (West and Tracy, 2009). A recent study further showed a sub-lethal effect on mating behavior and reproduction when both larvae and adults were exposed to ivermectin (van Koppenhagen et al., 2020). The predatory diet of adult yellow dung flies makes this species prone to contamination via insect prey that has been sprayed by or was otherwise in contact with insecticides such as spinosad. Therefore, the life cycle of this species is well-suited to investigate interacting carry-over effects of multiple chemicals ingested during different life stages.

We examined the separate and combined effects of ivermectin exposure during larval development and consumption of spinosad-contaminated prey (*Drosophila melanogaster*) at the adult stage, in the yellow dung fly with a common garden experiment with six treatments (3 ivermectin treatments × 2 spinosad treatments). We assessed development time, survival, body size, egg-to adult viability, and female fecundity. We hypothesized that yellow dung flies suffer from additive and interactive contamination effects whereby (1) both ivermectin and spinosad contaminations not only induce greater fitness costs than single contamination but also a greater costs than that predicted from the additive effects of the two contaminants, and (2) larval contamination (ivermectin) is more costly than adult contamination via prey (spinosad). The results of this experiment are important for our understanding of real-life scenarios of the effects of multiple pollutants on biota.

Methods

Study species

143 The yellow dung fly (*Scathophaga stercoraria*; Diptera: Scathophagidae) is a coprophagous fly
144 common throughout the northern hemisphere that is often found near cattle pastures (Fig. 1). In
145 Central Europe, the species has a spring and an autumn flight season, while it disappears during
146 the summer due to its sensitivity to high temperatures (Blanckenhorn, 1998; Blanckenhorn,
147 2009). The larvae are coprophagous whereas adults prey on small flying insects (Gibbons, 1980;
148 Blanckenhorn and Viele, 1999). Prey are a necessary source of protein for the females to produce
149 eggs and for males to produce sperm (Foster, 1967). Depending on the temperature, larval
150 development lasts 3-5 weeks and the adults take 5-15 days to become sexually mature
151 (Blanckenhorn and Henseler, 2005).

152 *Fly collection and breeding*

153 The individuals of our stock population used for this experiment stemmed from flies originally
154 caught in Appenzell, Switzerland (47°23'55"N, 8°34'39"E), and held in the laboratory for at
155 least 2 generations. More than sixty couples were collected in the field and then transported to
156 the laboratory in plastic tubes containing fresh dung, sugar, and water. Once in the lab, the flies
157 were provided with enough *Drosophila melanogaster* for the females to lay eggs. Each fly in our
158 stock population was kept individually in 100 mm glass bottle with sugar, water and it was
159 provided with >40 *Drosophila* two times a week . The flies were transferred into a new clean
160 bottle every week and then, randomly paired to generate the next generation. All flies were kept
161 in a climate chamber at constant conditions (18°C; 60% r.h.; 14L/10D).

162 *Dung preparation*

163 technical ivermectin (Chemical Abstracts Service no. 70288-86-7),.
164

165 The dung used for the experiment was originally collected from grass-fed and ivermectin-free
166 cattle. Their dung was brought to the laboratory, homogenized and frozen at -80°C for several
167 weeks. To assess the effect of technical ivermectin (with a purity of 94% for ivermectin B1a and
168 2.8% for ivermectin B1b; supplied by Paul Cooper, Merial; CAS118 No. 70288-86-7) on
169 foraging yellow dung fly larvae, three dung treatments were prepared: a control treatment (C0)
170 plus two ivermectin concentrations (C1=12 µg ivermectin/kg and C2=24 µg ivermectin/kg wet
171 dung, [0.36 and 0.72 mg ivermectin/50ml acetone, respectively]). These concentrations were
172 previously determined experimentally based on range-finder survival-concentration curves
173 (Supplementary material; van Koppenhagen et al., 2020). The contaminated dung was prepared
174 before the experiment by adding a solution of ivermectin dissolved in acetone, and was then kept
175 overnight at room temperature for the solvent to evaporate (Römbke et al., 2009).

176 *Ivermectin and spinosad contamination*

177 For the ivermectin contamination scenario of yellow dung fly larvae (Fig. 2), eggs were collected
178 from 58 mated females. The total number of eggs laid by each female (20-80 eggs) was split
179 evenly across the three treatments and then transferred into dung pots containing sufficient dung
180 (>45g of dung) to avoid competition and food shortage (Hellriegel and Blanckenhorn, 2002). A
181 total of 2394 eggs were randomly distributed across 174 dung pots of 3 larval ivermectin
182 treatments (58 replicates each). All pots were labeled individually with a code indicating the
183 treatment and egg laying date. The larvae were then reared in a climate chamber at 18°C, 60%
184 r.h., and 14L/10D. After roughly 2 weeks, dung pots were checked daily for newly emerged
185 individuals. Larval development time, egg-to-adult viability, and body size were subsequently
186 scored. Hind tibia length, a common surrogate of body size (Ward, 1998), was measured using
187 ImageJ v. 1.8.0_112 (NIH, Bethesda, MD, USA).

188 To mimic the more complex double-contamination scenario that adult yellow dung flies might
189 experience in the field, newly emerged adult individuals of each ivermectin treatment were
190 additionally exposed to the insecticide spinosad via consumption of contaminated food (Fig. 2).
191 Emerging adult flies were separated individually into 100 ml glass bottles containing sugar and
192 water that were plugged with foam stoppers to avoid cannibalism. Every day, roughly half of all
193 emerging adult flies of each larval treatment was supplied with uncontaminated *D. melanogaster*
194 prey while the other half received *Drosophila* contaminated with spinosad (Fig. 2, bottom). We
195 used 44.2% spinosad (480 g/l) from Renovita Wilen GmbH, which was diluted to 0.02 % with
196 distilled water based on the concentration permitted by the government (psm.admin.ch/) for
197 application to Swiss agricultural fields against fruit fly pests such as *Drosophila suzukii*, and
198 sprayed it directly onto adult of *D. melanogaster*. In total, we had six treatments corresponding
199 to three larval ivermectin treatments (control[C0], low concentration [C1], and high
200 concentration [C2]) crossed with two adult spinosad (control[C] and spinosad[S]) treatments
201 [C0-C (N=91), C0-S (N=47), C1-C (N=71), C1-S (N=59), C2-C (N=83), C2-S (N=53)] (Fig. 2).
202 Adult yellow dung flies were fed twice a week with about 50 *Drosophila* until they were
203 sexually mature.

204 To assess the fitness consequences of ivermectin and spinosad contamination on yellow dung
205 flies, we scored the first clutch laid by each female that emerged from each ivermectin treatment
206 and was subsequently exposed to spinosad-contaminated prey or not. Males and females were
207 subsequently paired randomly for copulation within ivermectin / spinosad treatment
208 combinations, and then transferred to a new glass containing dung at the time of copulation. We
209 carefully selected individuals from different unrelated parents to avoid potential negative effects
210 of inbreeding. After copulation, the male was removed from the bottles to avoid subsequent male

harassment and foster egg-laying. The occurrence of eggs was checked daily and the number of eggs recorded. Egg hatching success was measured by assessing the hatching rate of 10 eggs placed on filter paper humidified with fresh dung that was put on top of the dung in the pots so larvae could crawl into the dung. Afterwards the number of empty eggshells was scored using a binocular microscope (Leica MS5), yielding an estimate of egg hatching rate. Emergence success (i.e. egg to adult survival) was measured by dividing the total of emerged flies by the total number of eggs (or hatched larvae). Both these viability indices allowed us to partition the mortality between the egg and larval/pupal stages. Body size of the adult flies was later measured digitally based on the hind tibia.

Statistical analysis

All statistical analyses were carried out with R 3.5.1 (R Development Core Team, 2019). The sole effect of ivermectin contamination on sex-specific development time (log-transformed) and body size of the focal flies was assessed using two-way ANOVA. The combined effects of ivermectin and spinosad on clutch size (log-transformed) were tested with a two-way ANOVA additionally controlling for female body size as covariate, thus assessing both main and interactive effects. To further analyze offspring body size, we also used a three-way ANOVA including ivermectin, spinosad, and sex as main effects with all two- and three-way interactions. These interactions allowed us to detect potential synergistic (positive interaction) or antagonistic effects (negative interaction). Sexual size dimorphism (SSD) was calculated as the difference in hind tibia length of males and females within families (SSD=male-female) as revealed by Tukey contrasts. For each analysis with significant main effects we carried out a Tukey post-hoc test to compare pairwise combinations of treatment levels. Emergence success of the parents and offspring as well as egg viability were analyzed using a logistic regression model again

controlling for female body size. For each logistic regression, we conducted posthoc Tukey test with the *glht* function of the multcomp package (Hothorn et al., 2008b)to perform pairwise comparison between levels of the main effects. All values presented below are mean ± SD.

Results

Developmental responses to ivermectin contamination

Ivermectin treatment and sex had significant effects on development time of the flies (Fig. 3a). Consistently across treatments, females always emerged earlier than males (main sex effect in the ANOVA: $F_{1,138} = 54.9$, $P < 0.0001$). The individuals reared in the control treatment emerged earliest (mean ± SD: 24.35±1.2 d), followed by those raised in the ivermectin treatments (25.71± 1.3 d and 26.7±1.6 d for C1 and C2 treatments, respectively; ivermectin main effect: $F_{2,138} = 32.5$, $P < 0.0001$). On average across the sexes, the development time increased by 5.6% under C1 and by 9.6% under C2, although a Tukey test revealed that development time did not differ significantly between the two ivermectin concentrations (C1 and C2) ($P = 0.15$).

Egg-to-adult viability differed between ivermectin treatments (ivermectin main effect: $\chi^2 = 14.57$, $df = 2$, $P = 0.0006$; Fig. 3b). The mean emergence success in the three treatments was 0.82±0.17, 0.75±0.12, and 0.71±0.18 for the control, C1, and C2 treatments, respectively. Tukey test revealed that individuals from the control treatment showed significantly higher emergence success than both ivermectin treatments, but there was no significant difference between the two ivermectin concentrations (C1 and C2; P=0.6).

Body size at emergence differed significantly between the three ivermectin treatments (main ivermectin effect: $F_{2,291} = 109.1$, $P < 0.0001$) (Fig. 4a). Males were larger than females in this species across all treatments ($F_{1,291} = 804.2$, $P < 0.0001$). There was a significant interaction

between ivermectin and sex (ivermectin×sex: $F_{2,291} = 3.36$, P =0.03), indicating that the male-biased sexual size dimorphism (SSD) declined in C2 (Fig. 4b). This change in SSD was the result of a greater decline in male than female body size (11.6% vs. 9.8% with respect to the control).

Effects of ivermectin and spinosad on reproductive traits

Ivermectin significantly lowered female clutch size (log-transformed and corrected for female body size via ANCOVA; main effect: $F_{2,87} = 3.98$, P = 0.02; Fig. 5), although there was no significant difference between C1 and C2 (Tukey test: P = 0.48). Relative to the 42.34±21.46 eggs in the control treatments, feeding on spinosad-contaminated prey reduced clutch size on average by 27.4% (main spinosad effect: $F_{1,87} = 4.13$, P = 0.04), although a Tukey test did not show any significant spinosad effect within each ivermectin treatment (P>0.05). There was no significant interaction between ivermectin and spinosad on clutch size (P = ANOVA: $F_{2,87} =$ 1.34, P = 0.26).

Egg hatching success was also affected by both ivermectin and spinosad (Fig 6a). The average hatching rate in the control (without ivermectin and spinosad) was 0.84±0.23 (N=35). Ivermectin caused a decline in egg hatching success by 9.5% in C1 (0.76±0.32) and 34.5% in C2 (0.56±0.38) (main effect: $\chi2 = 59.71$, df = 2, P<0.0001). Spinosad decreased egg hatching success by 18.1% from an average of 0.78±0.31 to 0.64± 0.35 (main effect: $\chi2 = 20.87$, df = 1, P<0.0001) (Fig. 6a). Again, there was no significant interaction between ivermectin and spinosad (P=0.20).

The average offspring emergence success in the control treatment was 0.78± 0.25 (N = 35 families). Ivermectin reduced offspring emergence (main effect: $\chi2 = 311.98$, df = 2, P >0.0001)

to 0.65± 0.25 in C1 (N = 27) and 0.55±0.21 in C2 (N = 32), whereas spinosad reduced emergence success by 19.4% (average across all ivermectin treatments) ($\chi 2 = 61.10$, df = 1, P >0.0001). In addition, there was a significant interaction between ivermectin and spinosad ($\chi 2 = 10.58$, df = 2, P=0.005). This interaction resulted from a greater difference in emergence success between the spinosad treatments of offspring born from parents raised in C1 compared with those raised in the control and C2 (Fig. 6b).

Offspring body size was unaffected by ivermectin ($F_{2,228} = 1.31$, P = 0.27) or spinosad ($F_{1,228} = 0.33$, P = 0.56) (Fig 7a), but there was a significant interaction ($F_{2,228} = 5.74$, P = 0.003). This interaction was mediated by a significant difference in body size between the control and the C1 ivermectin concentration with no spinosad contamination (Tukey test: P = 0.009) combined with the absence of differences between any other such pairs (P > 0.05). As above, males were significantly larger than females ($F_{1,228} = 553.5$, P < 0.0001), while male-biased sexual size dimorphism did not change across treatments (non-significant three-way interaction between ivermectin, spinosad and sex: $F_{2,228} = 1.05$, P = 0.35; Fig 7b).

Discussion

Most research investigating the lethal and sublethal effects of toxic substances is based on single chemicals. However, species are exposed to a wide range of chemicals in the wild that might jointly affect them at different stages of their lives. These complex eco-toxicological scenarios combining different sources of contamination with potentially direct and transgenerational effects on the life history of organisms have not yet been studied sufficiently. We here experimentally applied a contamination scenario commonly experienced by natural populations of invertebrates and vertebrates in the widespread yellow dung fly, a common decomposer in north-temperate agricultural landscapes. The insect was exposed to the parasiticide ivermectin

during its larval stage and to the insecticide spinosad during its adult stage. We found alarming additive effects of these two substances on various key life-history and reproductive traits that are correlated with individual fitness, as well as transgenerational effects on their offspring. There were also synergistic negative effects of both contaminants on the offspring emergence success and their body size. These results suggest major direct and indirect impacts of these chemicals on natural insect populations at the local, regional and global scales (Hallmann et al., 2014).

Ivermectin effects on larvae

Exposure of juvenile yellow dung flies to ivermectin at environmentally relevant concentrations increased mortality before emergence, as was known before to occur in the laboratory and the field (Römbke et al., 2009; Jochmann and Blanckenhorn, 2016). Ivermectin prevents larvae from pupating, as shown by Strong and James (1993) and West and Tracy (2009) in the yellow dung fly, and by Cruz Rosales et al. (2012) in the dung beetle *Euoniticellus intermedius*. Strong and James (1993) reported that half of the larvae were prevented from pupation when raised in 0.015 mg kg^{-1} ivermectin. Ivermectin further prolonged the flies' development time, an important life-history trait that determines the fitness of yellow dung flies in the wild because of the ephemeral nature of its habitat (fresh dung dries relatively fast: Blanckenhorn, 1998), and also reduced their final adult body size. The latter implies slowed growth rates, probably due to altering neurotransmission pathways (Fritz et al., 1979). Similar results were found by Römbke et al. (2009) and van Koppenhagen et al. (2020) for the same species. Interestingly, ivermectin decreased the magnitude of sexual size dimorphism (SSD) (the difference in male and female body size) by disproportionately reducing the size of the larger sex, here the male. As SSD is condition-dependent in many insects (Rohner et al., 2018), it is probable that males could not

allocate a substantial part of their energy into accruing body size in a highly contaminated habitat, thus limiting their growth plasticity (Blanckenhorn, 1998). This should have a considerable effect on sexual selection, because larger males typically have more energy reserves and are more vigorous (Jann et al., 2000; Blanckenhorn et al., 2003).

Ivermectin effects on adults

Clutch size of the emerged adults was reduced by 25% to 34% in our two ivermectin treatments, as also found for the same species by van Koppenhagen et al. (2020). Such negative effects of ivermectin on fecundity have been observed in a wide range of insects (Desneux et al., 2007), e.g. the dung fly *Sepsis punctum* (Conforti et al., 2018), the dung beetle *Euoniticellus intermedius* (Cruz Rosales et al., 2012), or the fly *Musca nevilli* (Krüger and Scholtz, 1995). The process driving the reduction of egg number in response to ivermectin is likely related to delayed egg development or prevention of vitellogenesis (the arrest of yolk deposition within oocytes) (Martínez et al., 2017).

Transgenerational effects of ivermectin

While ivermectin reduces emergence success (egg to adult viability) of adults, we further found that the adults that emerge from contaminated environments produced offspring that also experience greater mortality than the offspring of adults when raised in uncontaminated environments. Interestingly, both ivermectin concentrations similarly affected the larval development and mortality of parents but showed a greater effect on offspring in C2 than C1. This finding highlights a transgenerational carry-over effect of parental exposure to toxic substances. Similar parent-to-offspring carry-over effects of toxic substances on emergence success were obtained in beetles (Baena-Díaz et al., 2018; Müller et al., 2019). By analyzing egg

346 hatching success, we could disentangle mortality occurring during the egg vs. larval stage. Eggs
347 had high hatching rates in the control and in the low ivermectin concentration (C1) but declined
348 severely (34.5%) at the higher concentration (C2). Previous studies have shown a negative effect
349 of ivermectin on egg hatching success in other flies (McGarry, 1988). The relatively lower adult
350 emergence we found at the low concentration (C1) demonstrates that both larval mortality and
351 egg hatching failures contributed to adult emergence failure, as the considerably lower
352 emergence success of adults at the high concentration (C2) was mainly due to egg mortality. This
353 suggests that even if the offspring are not exposed to ivermectin while the parents were,
354 vitellogenesis is still disrupted by some mechanisms operating on, for instance, polyamine
355 synthesis, which is responsible for yolk formation in some insects (Kogan and Hagedorn, 2000).

356 In contrast, parental exposure to ivermectin did not affect offspring body size of either sex. This
357 result is similar to that found for the dung beetle *E. intermedius* (Baena-Díaz et al., 2018). Given
358 that the offspring dung was not contaminated by ivermectin, it is therefore likely that the
359 surviving offspring ultimately had similar metabolic rates as those produced by uncontaminated
360 parents. More information on the competitive ability and lifetime reproductive output of these
361 flies is needed for stronger conclusions about these transgenerational effects (Jann et al., 2000),
362 and more studies are required to unravel whether parental investment or epigenetic processes
363 play a role in maintaining offspring body size and fitness in ivermectin-contaminated
364 environments (Baena-Díaz et al., 2018). Van Koppenhagen et al. (2020) demonstrated that adult
365 yellow dung flies of both sexes feeding on ivermectin-contaminated sugar also experienced
366 negative effects on several life-history, behavioral and reproductive traits, most notably a
367 reduction in male fertility and, specifically, testis size (even when controlling for female
368 contamination). Other male fertility traits, such as sperm number and quality, could also be

reduced by contamination (Conforti et al., 2018). When investigating female fecundity, Conforti et al. (2018) showed for sepsid flies that contamination reduced the number of eggs laid and offspring emerged.

Spinosad effects on adults

Although spinosad has been shown to be relatively safe for beneficial non-target insects (Williams et al., 2003; Thomas and Mangan, 2005), studies have highlighted some negative effects on natural pest enemies such as beetles, lacewings, and earwigs (Cisneros et al., 2002), either at the larval or the adult stage (Galvan et al., 2005). In our study adult emergence (i.e. egg to adult survival) declined when the parents ingested prey contaminated with spinosad (both parents were contaminated). This finding suggests that contamination of parental food affects the ontogeny of their offspring during maturation, ultimately reducing their survival probability. Whether the factors driving egg or larval mortality originate from the father and/or mother is unsure. Our results demonstrate that offspring quality can be reduced via parental effects when parents ingest contaminants such as spinosad.

Spinosad and ivermectin effects on offspring

In our study, spinosad sprayed on prey ingested by adult yellow dung flies produced a reproductive cost on clutch size and egg viability additional to that of ivermectin. This finding strengthens the hypothesis that spinosad affects mechanisms behind egg production and egg fertility. Fecundity of the moth *Helicoverpa armigera* was lowered by spinosad when administered at the larval stage (Wang et al., 2009). This is in line with studies investigating the effect of spinosad on female fecundity in lacewings (Nadel et al., 2007), beetles (Galvan et al., 2005), and mites (Villanueva and Walgenbach, 2005). Nevertheless, various other studies show

391 variability in the effect of spinosad on these reproductive traits, depending on taxa and life stage
392 (Davey et al., 2001; Viñuela et al., 2001; Medina et al., 2003; Biondi et al., 2012), highlighting
393 the complexity of ecotoxicological impacts of this chemical on biotic processes.

394 Emergence success (i.e. egg to adult survival) suffered from both additive and interactive effects
395 of ivermectin and spinosad depending on ivermectin concentration. The synergistic effects of
396 ivermectin and spinosad were detected at low ivermectin concentration (C1), whereas merely
397 additive effects were observed at high ivermectin concentration (C2). Most notably, the
398 synergistic effect induced mortality that was quite similar to that observed at high ivermectin
399 concentration. This finding is interesting given that the chemical interaction occurs after the
400 bioaccumulation of different pesticides at different life stages. The fact that no synergistic effect
401 was observed for egg viability reveals that the increased mortality occurred either during the
402 larval stage or pupation. Synergistic effects after simultaneous application of different pesticides
403 have been observed for various chemicals in diverse insects (Marcus and Lichtenstein, 1979; El-
404 Guindy et al., 1983; Ishaaya, 1993; Hsu et al., 2004). Dose-dependent synergism between
405 chemical mixtures have been observed in studies on bees (Zhu et al., 2014), where the interaction
406 often occurs at high doses, while in a study on earthworms the interaction was detected at low
407 concentrations (Chen et al., 2015). The absence of synergism at the higher ivermectin
408 concentration (C2) in our study might be due to physiological responses that occurred only at
409 that concentration, thus perhaps precluding an interactive effect beyond the independent primary
410 action of ivermectin and spinosad. Further investigations are needed to scrutinize the interaction
411 between physiological responses and chemical exposure (Hernández et al., 2013), and more
412 analyses are required to unravel the underlying physiological processes involved in synergistic
413 effects of different contaminants.

Offspring body size was not strongly sensitive to parental contamination by ivermectin or spinosad. Thus our results suggest that offspring development is more prone to toxic contamination during the ontogeny of the parents than during their adult life. The slight increase of female offspring size from parents contaminated by both spinosad and ivermectin could result from a beneficial hormesis effect at lower doses of both contaminants, which could have enhanced some life history trait such as body size. Such effects have been documented in different studies (Guedes et al., 2010; Tricoire-Leignel et al., 2012) . A similar increase was observed in progeny wing length of the mosquito *Aedes aegypti* after spinosad contamination of the mother (Antonio et al., 2009). It is possible that low levels of toxicity (by ivermectin) fosters parental investment in progeny resistance to contamination (Szabó and Bakonyi, 2017), but this remains to be tested specifically.

Interestingly, our study highlights a biological aspect that has not been widely discussed in ecotoxicological studies. The mode of feeding of predatory insects differs such that some species eat parts or the entire body of insects, while others consume the internal liquids only (blood-sucking), leaving the exoskeleton of the prey largely untouched. Yellow dung flies feed by biting a hole into the body (often the head) of their prey and regurgitating some enzymes into it, which are later sucked up again (i.e. extra-intestinal feeding: Gibbons, 1980; Swaddle, 1997) . Thus they largely belong to the latter category, but still suffered from feeding on contaminated prey. This is interesting because spinosad is said to be more toxic through consumption than contact (Tillman and Mulrooney, 2000). We therefore suggest that either prey handling alone leads to spinosad contamination, or spinosad infiltrates the body of the prey (through exoskeleton penetration or consumption) and is subsequently ingested by the predators.

Further attention should be devoted to the understanding of the prevalence and consequences of synergistic effects of pesticides on beneficial organisms. In fact, among the most exposed species in the wild are those that provide vital ecosystem services such as pollination or pest control. Future research should be carried out on whether the synergistic effects of different contaminants persist, increase or decrease across trophic levels, and on the role of climate change in shaping the biotic responses to these pesticide interactions.

Conclusion

In natural habitats, species are exposed to several potentially interacting pesticides, such as the parasiticide ivermectin and the insecticide spinosad, which are widely applied by farmers worldwide. Our results show strong evidence of largely independent negative, but sometimes also synergistic effects of ivermectin and spinosad on multiple life-history traits of the common yellow dung fly, including transgenerational carry-over effects on the offspring of contaminated parents. These findings suggest that pollution from multiple sources will have cumulative and synergistic effects on population dynamics and phenotypic traits of natural insect populations and likely other organisms. The persistence of toxicity through generations is something that should be considered carefully by environmental and human health authorities as well as policymakers.

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Figure captions

Figure 1. Male yellow dung fly (*Scathophaga stercoraria*) in the wild (a) feeding on a prey item and (b) copulating with a female (photo credit: Rassim Khelifa).

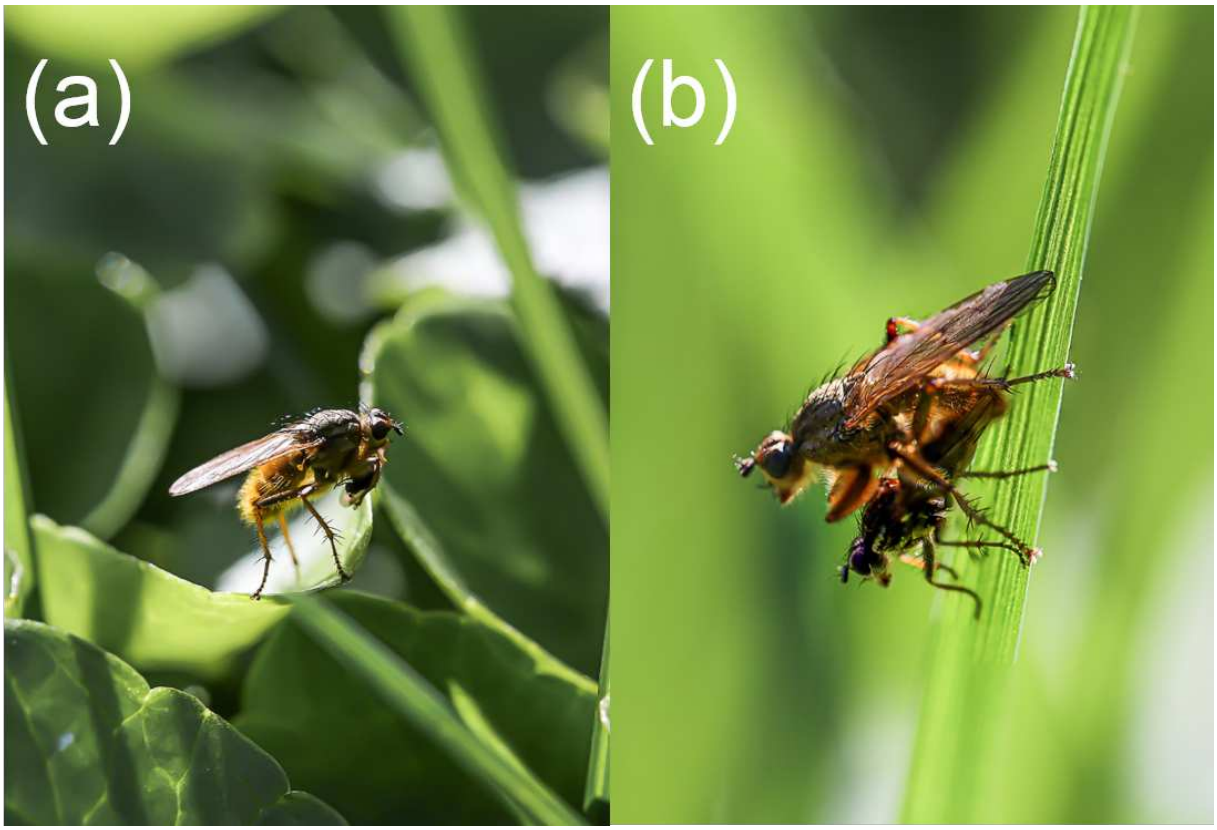


Figure 2. Graphical representation of the fully-factorial experimental design to examine the combined effects of ivermectin and spinosad on yellow dung fly reproductive traits (*Scathophaga stercoraria*) at the juvenile and adult stages. Phase 1 (3 treatments) tests for the effects of ivermectin contamination on juvenile life-history traits (egg-to-adult survival, development time, body size). Phase 2 (2 treatments) uses individuals emerging from the three larval treatments of phase 1 to test for additional effects of the consumption of spinosad-contaminated prey on adult reproductive traits (clutch size, egg hatching success, egg-to-adult survival, and body size of the offspring).

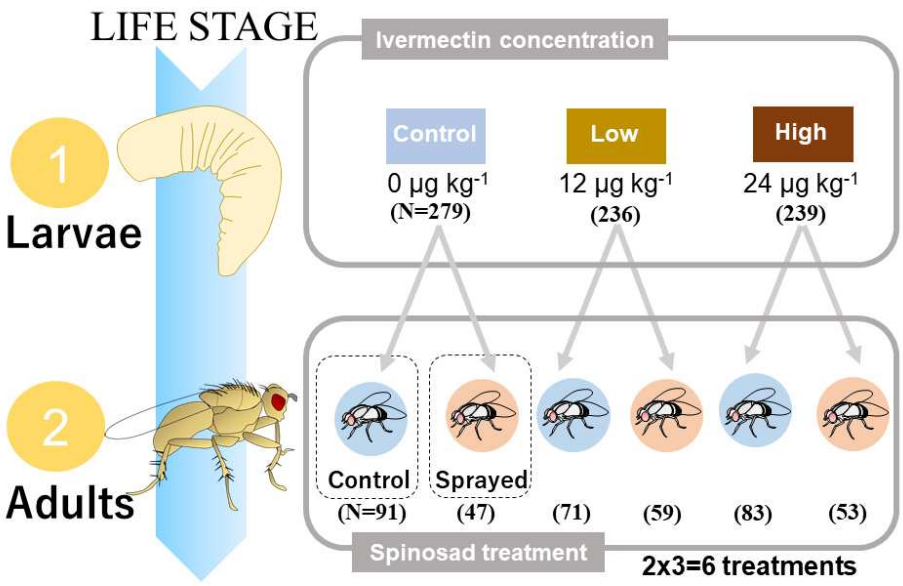
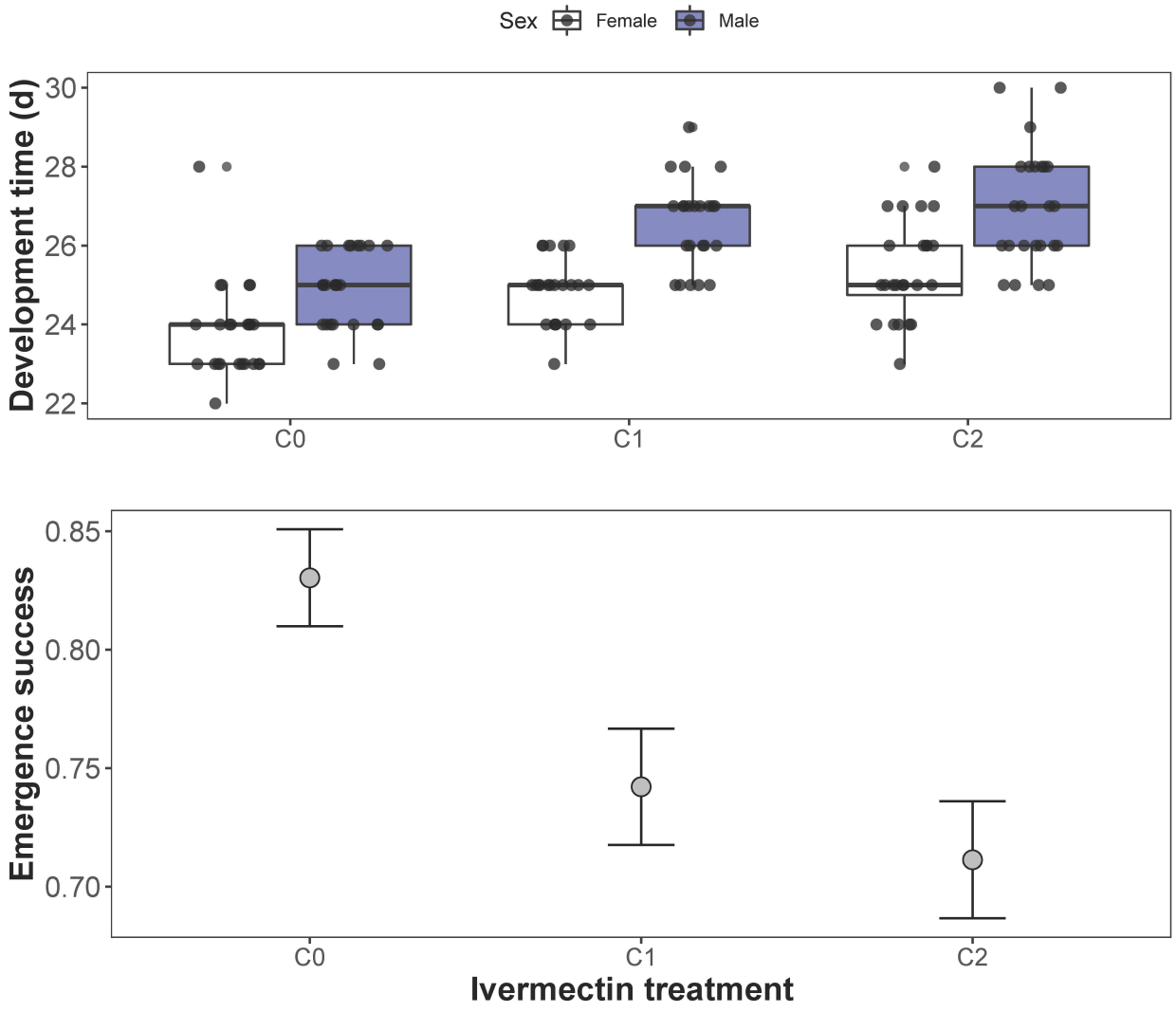
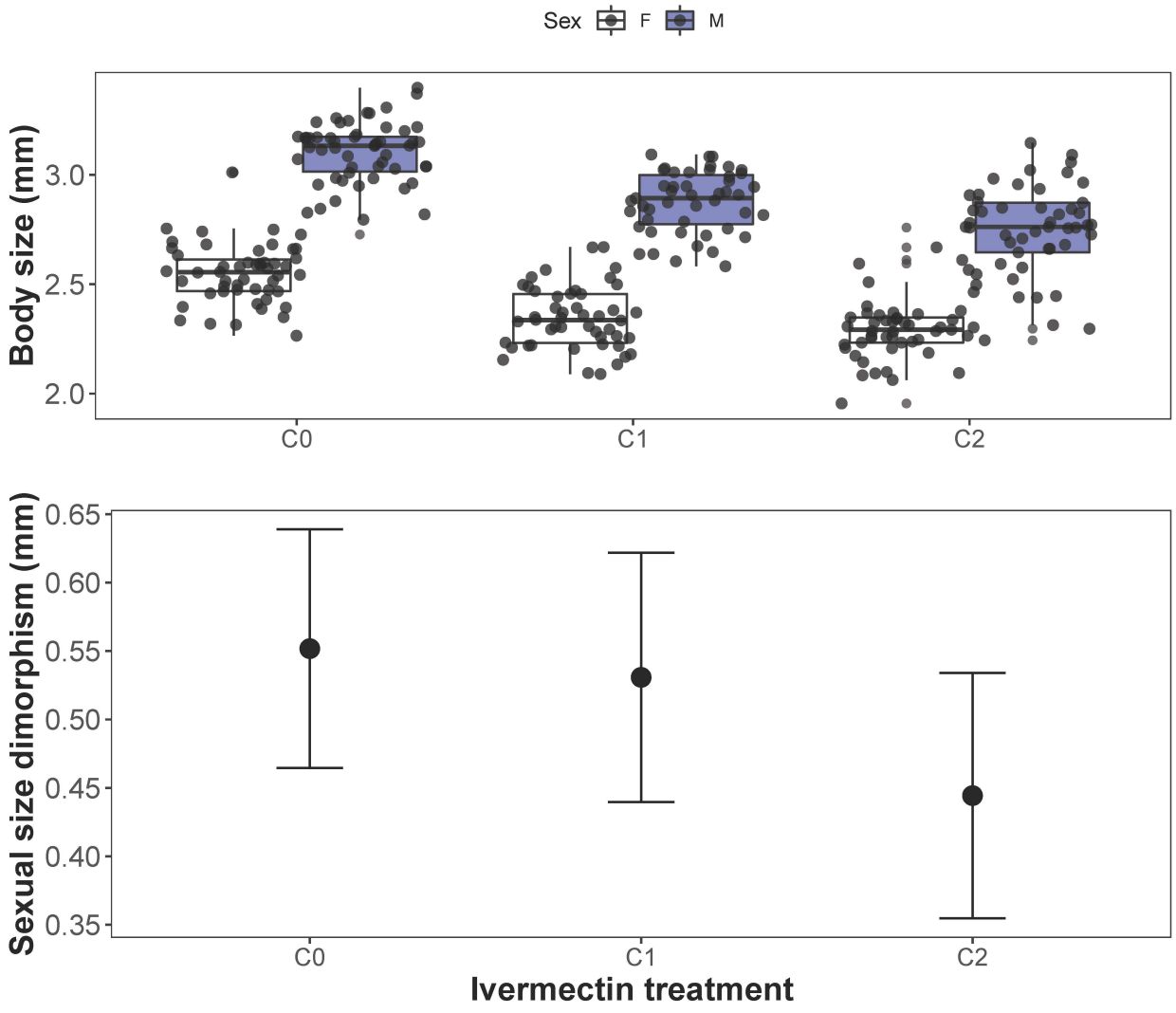


Figure 3. Boxplot and error-bar plot showing the effect of ivermectin on (a) development time and (b) emergence success (egg-to-adult viability) of yellow dung flies (*Scathophaga stercoraria*). C0 is the control, C1 is the low (12µg kg^{-1}), and C2 is the high ivermectin concentration (24µg kg^{-1}). Error bars are 95% confidence intervals. Colors refer to sex (male [blue], female [clear]).



730 **Figure 4.** Box and error bar plots showing the effect of ivermectin on (a) body size (hind tibia)
731 and (b) sexual size dimorphism [SSD] of yellow dung flies (*Scathophaga stercoraria*). C0 is the
732 control, C1 is the low (12µg kg^{-1}), and C2 is the high ivermectin concentration (24µg kg^{-1}). Error
733 bars are 95% confidence intervals. Colors refer to sex (male [blue], female [clear]).



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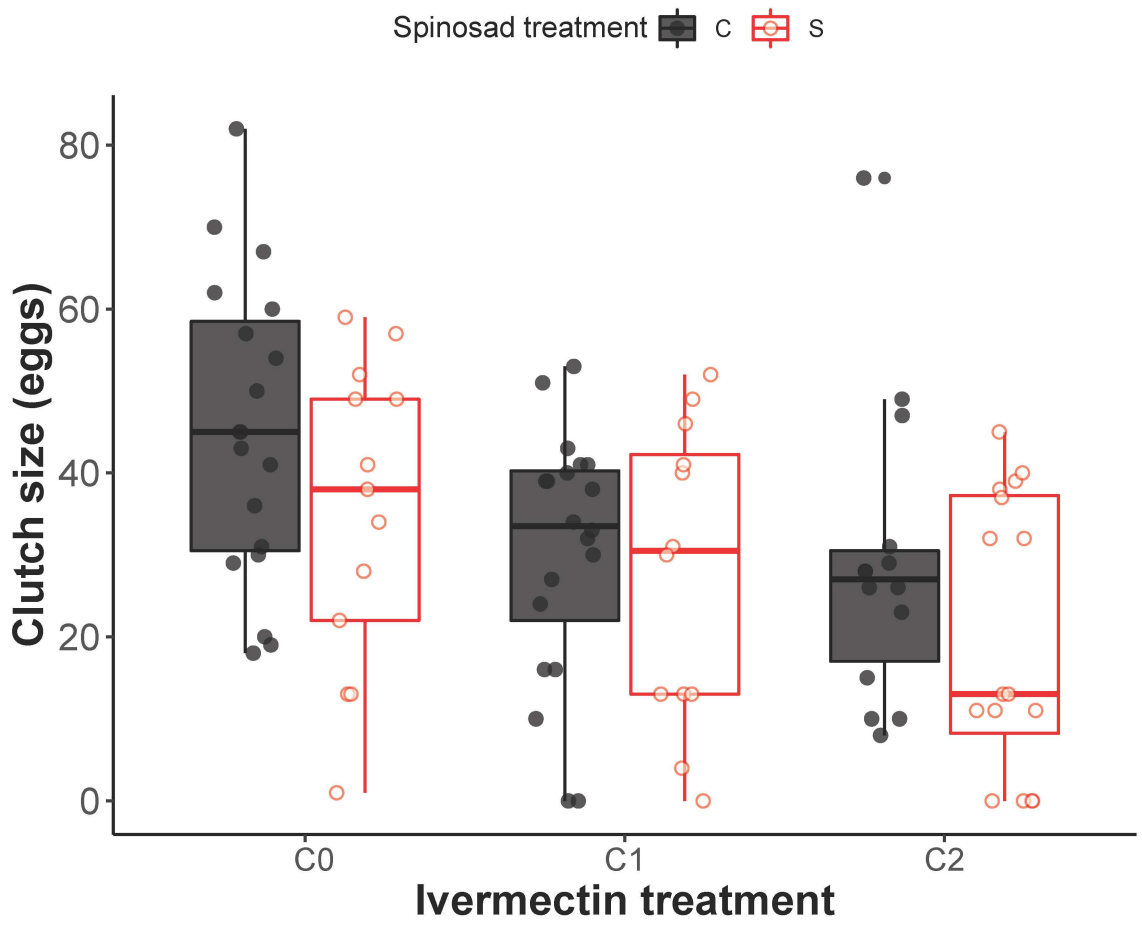
736 **Figure 5.** Boxplots showing the effects of ivermectin and spinosad on the first clutch size of
737 yellow dung fly females (*Scathophaga stercoraria*). C0 is the control, C1 is the low
738 concentration (12µg kg^{-1}), and C2 is the high ivermectin concentration (24µg kg^{-1}). Colors refer
739 to spinosad treatments (Control: unsprayed [black], Spinosad: sprayed [red]). Error bars are 95%
740 confidence intervals.



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743 **Figure 6.** Error bar plots depicting the effects of ivermectin and spinosad on (a) egg hatching
744 success and (b) offspring emergence success of yellow dung flies (*Scathophaga stercoraria*). C0
745 is the control, C1 is the low ivermectin concentration (12µg kg^{-1}), and C2 is the high ivermectin
746 concentration (24µg kg^{-1}). Colors refer to spinosad treatments (control: unsprayed [black],
747 spinosad: sprayed [red]). Error bars are 95% confidence intervals.

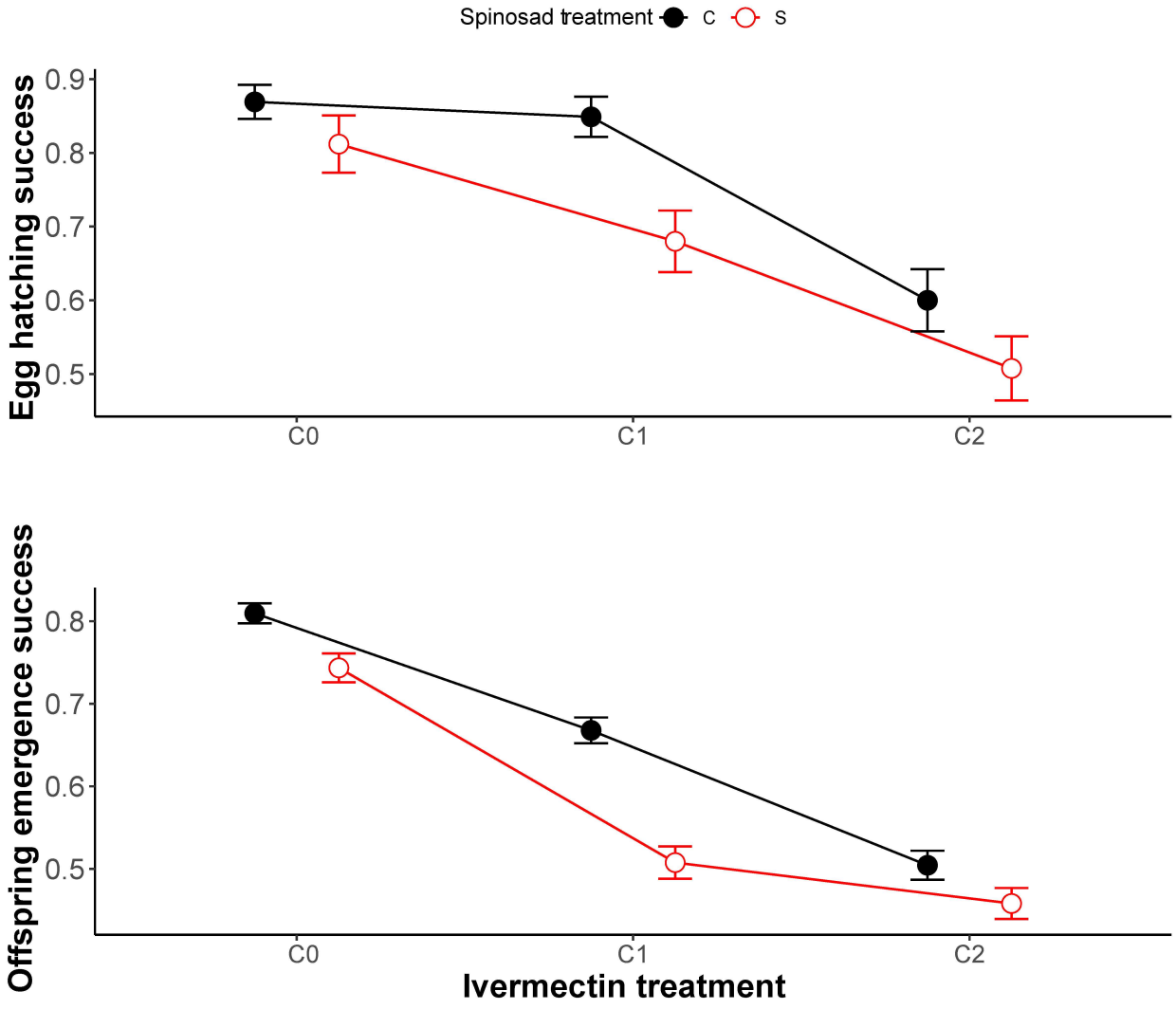

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Figure 7. Error bar plots showing the effects of ivermectin and spinosad on (a) body size (hind tibia) and (b) sexual size dimorphism of the offspring of contaminated yellow dung fly parents (*Scathophaga stercoraria*) growing up in uncontaminated dung. C0 is the control, C1 is the low concentration (12µg kg^{-1}), and C2 is the high ivermectin concentration (24µg kg^{-1}). Colors refer to spinosad treatments (control: unsprayed [black], spinosad: sprayed [red]). Shapes refer to sex (triangle: female, circle: male). Error bars are 95% confidence intervals.